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Regulation of LPA receptor function by estrogens

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Abstract

17β-Estradiol induced LPA_1 receptor desensitization in C9 cells stably expressing LPA_1 receptors and transiently expressing estrogen receptor α. Such desensitization was evidenced by a reduction in lysophosphatidic acid-mediated Ca^{2+}mobilization and it was associated to receptor phosphorylation and internalization. These effects of 17β-estradiol were rapid (taking place over 5 min) and were blocked by the estrogen receptor antagonist ICI 182780. Similarly, inhibitors of phosphoinositide 3-kinase (wortmannin and LY294002) and of protein kinase C (staurosporine and Gö 6976) blocked 17β-estradiol-induced LPA_1 receptor desensitization and phosphorylation. Confocal microscopy evidenced LPA_1 receptor internalization in response to 17β-estradiol treatment. Association between LPA_1 receptors and protein kinase C α was suggested by co-immunoprecipitation assays. Protein kinase C α was associated with LPA_1 receptors in the absence of stimulus and such association further increased in a dynamic fashion in response to 17β-estradiol. The results demonstrated that in C9 cells estrogens modulate LPA_1 action through estrogen receptor α with the participation of protein kinase C α and phosphoinositide 3-kinase.

Keywords: LPA_1 receptor; PKC; PI3K; 17-*β* estradiol; ERα; GPCR

1. Introduction

LPA (lysophosphatidic acid) was originally reported as one of the major growth factors in mammalian serum [1,2], and it is now recognized as an extracellular lipid mediator with a great variety of actions in diverse cell types. LPA is a potent inducer of cytoskeletal remodeling and cell migration, and a promoter of cell survival and proliferation [1,3–5].

This remarkable diversity of responses is explained by the fact that LPA action occurs via a large family of G protein-coupled receptors (GPCRs) with multiple downstream effectors. To date, five functional LPA receptors have been identified in mice and humans [6,7]: LPA_1 [8,9], LPA_2 [10], LPA_3 [11], LPA_4 [12,13] and LPA_5 [14,15]. Genetic deletion studies in mice have shown their essential biological functions [16–20]. LPA receptors have important participation in processes such as development, implantation and embryo spacing [20], organization of brain architecture and function [16,21], and initiation of neuropathic pain [22], among others. While essential roles for individual receptors have been observed, it has been somewhat surprising that more profound effects such as early embryonic lethality have not been observed in single or even double receptor knock-outs [17], in contrast with results of deletion studies for the key LPA biosynthetic enzyme, autotaxin, which produces embryonic lethality [18]. Functional redundancy of receptor subtypes likely explains such a paradox.

GPCR function is regulated by means of many different processes with different time frames. Receptor phosphorylation appears to be a cardinal initial event in the desensitization of these receptors and occurs mainly on serine and threonine residues located in the receptor's third cytoplasmic loop and/or C-terminal tail [23,24]. Receptor phosphorylation increases their affinity for a family of cytoplasmic proteins, known as

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arrestins [25], which attenuate signaling by blocking G-protein-receptor interaction [26]. In addition, arrestins act as adapters facilitating clathrin coated pit-mediated receptor endocytosis [26–30]. Internalized receptors are ultimately dephosphorylated by membrane-associated phosphatases [31,32], recycled back to the plasma membrane, or ubiquitin-targeted for degradation. Several lines of evidence support the hypothesis that receptor internalization is also required for resensitization [27,28,30,33].

Two major types of desensitization exist: homologous, and heterologous. Homologous desensitization, in which receptors occupied by agonists reduce their responsiveness, mainly involves receptor phosphorylation by G protein-coupled receptor kinases. Heterologous desensitization is induced by agents pharmacologically unrelated with the target, and mainly involves phosphorylation of receptors and other signaling entities by second messenger-activated kinases, such as protein kinase A and protein kinase C (PKC) [27,34–36].

Estrogen receptors (ERs) are nuclear transcription factors, which, when bound to agonists such as 17-β estradiol (E_2), modulate gene transcription. There are two estrogen receptor isoforms, these arising from different genes: ERα, and ERβ [37,38]. Despite the clarity with which ERs have been shown to act as transcription factors, many studies support the notion that not all biological effects of estrogens are accomplished via regulation of gene expression [39,40]. Estrogens induce rapid activation of diverse signal transduction pathways in time frames (sec to min) which are inconsistent with the possibility of being mediated by synthesis of RNA or protein; furthermore, these effects are usually insensitive to inhibitors of RNA and protein synthesis [41]. Rapid non-genomic responses to estrogens often occur via second messenger cascades, which in turn originate from signaling complexes located in close proximity to or at the plasma membrane (for review, see [42]). ERα directly associates with the p85α regulatory subunit of phosphoinositide 3-kinase (PI3K) in a hormone-dependent manner leading to PI3K/Akt signaling pathway activation inducing nitric oxide synthase activity [39,40].

Previous work at our laboratory has shown that ERα activation induces α_{1b}-adrenoceptor phosphorylation, desensitization and internalization through a signaling cascade involving PI3K and PKC δ activities [43]. In addition, we have shown that PKC stimulation induces LPA_1 receptor phosphorylation [44]. In this work, we tested whether E_2 could induce heterologous regulation of LPA_1 and further explored the molecular mechanisms involved.

2. Materials and methods

2.1. Materials

1-α-Lysophosphatidic acid (oleoyl-*sn*-glycero-3-phosphate) (LPA), 17-β estradiol (E_2), tetradecanoyl phorbol acetate (TPA), staurosporine, rottlerin, Gö 6976, wortmannin, and protease inhibitors were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). LY294002 was obtained from Calbiochem (La Jolla, CA, USA). ICI 182780 was from Tocris Cookson Inc (Ellisville, MO, USA). Sepharose-coupled protein A was from Upstate Biotechnology (Lake Placid, NY, USA). Nitrocellulose membranes were from Bio-Rad (Hercules, CA, USA). All primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), except the anti-EGFP, which was generated in our laboratory against GST-fused EGFP protein. Secondary antibodies were from Zymed (San Francisco, CA, USA), while chemiluminescence kits were obtained from Pierce (Rockford, IL, USA). Ham's F12 medium Kaighn's modification (F12K) and Dulbecco's modified Eagle's medium (DMEM) without phenol red were from In Vitro (México D.F, México.), fetal bovine serum, trypsin, antibiotics, reagents used for cell culture, Fura-2 AM (acetoxy-methyl-ester) and Lipofectamine™ 2000 were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). [^{32}P]Pi (8500–9120 Ci/mmol) was purchased from Perkin Elmer Life Sciences (Wellesley, MA, USA).

2.2. Cell culture

The rat hepatic epithelial cell line, C9, and the human breast cancer cell line, T47D, were cultured in F12K and RPMI media, respectively. Culture media was supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 0.25 μg/ml amphotericin B and cells were maintained at 37 °C under a 95% air, 5% CO_2 atmosphere. Growth medium was removed and replaced with DMEM without phenol red and without fetal bovine serum, 24 h prior to the experiments.

2.3. Molecular receptor characterization by RT-PCR

Total RNA was isolated using TRIzol® reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-PCRs were performed in a 50 μl reaction mixture consisting of 0.1 unit/μl RT AMV (avian myeloblastosis virus), 0.1 unit/μl *Tfl* DNA polymerase, reaction buffer AMV/*TF1*, 1 mM $MgSO_4$, 0.2 mM of each dNTP, 1 μM of each primer and 0.1 μg of total RNA. Samples were incubated at 48 °C for 45 min, followed by 2 min at 95 °C, and 35 cycles of 30 s at 95 °C, 30 s at 56 °C, and 2 min at 72 °C. We employed the primers reported by Moller et al. [45] and these were purchased from Sigma Chemical Co (St. Louis, MO, USA).

2.4. Intracellular calcium ($[Ca^{2+}]_i$) determinations

Cells were loaded with 2.5 μM of the fluorescent Ca^{2+}indicator, Fura-2/AM, in Krebs–Ringer-HEPES containing 0.05% bovine serum albumin, pH 7.4 for 1 h at 37 °C. Cells were washed three times to eliminate unincorporated dye. Fluorescence measurements were carried at 340 nm and 380 nm excitation wavelengths and 510 nm emission wavelength, with a chopper interval set at 0.5 s using an AMINCO-Bowman Series 2 luminescence spectrometer (Rochester, NY, USA). Intracellular calcium ($[Ca^{2+}]_i$) was calculated according to Grynkiewicz et al. [46].

2.5. Expression of LPA_1–EGFP in C9 Cells

The full-length cDNA encoding the mouse LPA_1 (generously given to us by Dr Kevin Lynch, Department of Pharmacology, University of Virginia, Charlottesville, VA, USA) was amplified and tagged at the C terminus with EGFP as a fusion protein [43]. C9 cells were stably transfected with the construct utilizing Lipofectamine™ 2000, following the manufacturer's protocol. Clones expressing high levels of EGFP-tagged LPA receptors were selected by resistance to geneticin (G418) as well as by fluorescence cell-sorting using FACSCalibur (BD Biosciences) and Cell Quest software (BD Biosciences). As negative control, C9 cells were also stably transfected with the pEGFP-N1 vector.

2.6. ERα transient expression in C9 cells

C9 cells that stably express LPA_1–EGFP were transfected with the human ERα kindly provided to us by Dr. A.J. Cooney, Baylor College of Medicine (Houston, TX, USA), using Lipofectamine™ 2000, following the manufacturer's protocol.

2.7. ERα–ECFP construction and transient expression in C9 cells

To generate the ERα fused to enhanced cyan fluorescent protein (ECFP) construct, the full-length human ERα was obtained from pT7BhERα plasmid by PCR amplification kindly provided by Dr. A.J. Cooney, Baylor College of Medicine (Houston, TX, USA). An XhoI site followed by ERα start nucleotide

sequence was designed in the forward primer, 5′-GGACTCAGATCTCGAGC-TATGACCATGACCCTCCA-3′. The reverse primer contained a BamHI site after a stop codon, 5′-GTAATTAGATGGATCCCTTCAGACCGT GGCAGGGA-AACC-3′. The amplified DNA product was subcloned into pCR® II-TOPO vector (Invitrogen Life Technologies, Carlsbad, CA, USA) and then re-isolated with the appropriate enzymes and subcloned into pECFP-C1 vector (CLONTECH Laboratories, Inc. Palo Alto, CA, USA). The construct was sequenced to verify that mutations did not occur during PCR amplifications. This receptor was fully functional as evidenced in estrogen-mediated gene expression experiments (Rocío García-Becerra and Fernando Larrea, unpublished data). C9 cells that stably express LPA_1–EGFP were transiently transfected with this construct utilizing Lipofectamine™ 2000, according to the manufacturer's protocol.

2.8. Immunoprecipitation and metabolic labeling of receptors

Cells approaching confluence growing in 35-mm plates were incubated in phosphate-free Dulbecco's modified Eagle's medium for 1 h and labeled with [^{32}P]P_i 100 μCi/ml in the same culture media for 4 h at 37 °C. Labeled cells were stimulated with agents as indicated, and were then washed twice with ice-cold phosphate-buffered saline and solubilized with 0.5 ml of ice-cold lysis buffer containing: 100 mM NaCl, 20 mM NaF, 10 mM sodium pyrophosphate, 50 mM Tris pH 8, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 10 mg/ml deoxycholic acid (sodium salt) and protease inhibitors (20 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μg/ml PMSF, 500 μg/ml bacitracin, 20 μg/ml benzamidine, 20 μg/ml pepstatin, 50 μg/ml trypsin inhibitor).

Plates were maintained on ice for 1 h and insoluble material was pelleted by centrifugation at 12000 ×*g* for 15 min at 4 °C. Supernatants were incubated overnight at constant agitation at 4 °C with 1:250 anti-EGFP antiserum and sepharose-coupled protein A. The following day, samples were centrifuged and the pellets washed three times with buffer WB1 (50 mM Tris HCl, 150 mM NaCl, 1% Triton X-100, 12 mM deoxycholic acid (sodium salt), pH 7.5), twice with buffer WB2 (50 mM Tris HCl, 500 mM NaCl, 0.1% Triton X-100, 1.2 mM deoxycholic acid (sodium salt), pH 7.5) and once with buffer WB3 (50 mM Tris HCl, 0.1% Triton X-100, 1.2 mM deoxycholic acid (sodium salt), pH 7.5). Finally the immune complexes were denatured by boiling on loading buffer (120 mM Tris pH 6.8, 4% SDS, 0.2% glycerol, 5% β-mercaptoethanol, 7 M Urea, 1 mM DTT and 10 mg/ml bromophenol blue) and subjected to SDS/PAGE. The gels were dried and receptor phosphorylation was assessed using a Molecular Dynamics PhosphorImager (Sunnyvale, CA, USA) and the Imagequant software (Amersham Biosciences; Piscataway, NJ, USA).

2.9. Co-immunoprecipitation studies

LPA_1–EGFP receptors were immunoprecipitated as described previously, with minor modifications. In brief, following treatment with ICI 182780, inhibitors and/or E_2, cells were washed with ice-cold phosphate-buffered saline and lysed for 1 h with ice-cold lysis buffer without deoxycholic acid. Lysates were centrifuged at 12,700 ×*g* for 15 min and the supernatants were incubated overnight at 4 °C with the anti-EGFP antiserum and protein A-Sepharose. After one wash with 50 mM HEPES, 50 mM NaH_2PO_4, 100 mM NaCl, pH 7.4, and 0.1% Nonident P40, the immune complexes were denatured by boiling in sodium dodecyl sulfate-sample buffer containing 5% β-mercaptoethanol, and subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electrotransferred to nitrocellulose membranes and immunoblotting was performed. Incubation with PKC α-or PKC ε-selective antibodies was performed for 12 h at 4 °C and with the secondary antibody for 30 min at room temperature. Super signal-enhanced chemiluminescence kits were used, exposing membranes to X-Omat X-ray films; all co-immunoprecipitations were assessed by densitometric analysis.

2.10. Western blot assays

Cells were washed with ice-cold phosphate-buffered saline and lysed for 1 h on ice as previously described. Lysates were centrifuged at 12,700 ×*g* for 15 min and proteins in supernatants were quantified. In all immunoblots, equal amounts of protein (70 μg) were separated by electrophoresis on 10% SDS-PAGE. Proteins were electrotransferred to nitrocellulose membranes and immunoblotting was performed. Incubation with ERα or ERβ selective antibodies or anti-EGFP serum was conducted for 12 h at 4 °C and with the secondary antibody for 30 min at room temperature. Super signal-enhanced chemiluminescence kits were used exposing the membranes to X-Omat X-ray films.

2.11. Confocal microscopy

Confocal images were obtained using a OLYMPUS Fluoview FV 1000 confocal system (Olympous corporation, Shinjuku Monolith, Tokyo, Japan) attached/interfaced to an OLYMPUS IX81 inverted light microscope with a 40× glycerol-immersion objective. EGFP was excited using the 488 nm line of a krypton/argon laser and the fluorescence emitted was detected with a 515–540 nm band pass filter. ECFP was excited employing the 405 nm line of a krypton/argon laser and the fluorescence emitted was detected with a 425–476 nm band pass filter. All images were obtained using numeric aperture 1.3 and the same laser percentage, iris aperture and gain. Operating the laser at a low power setting (97–99% attenuation) substantially reduced photobleaching and photodamage. Confocal images were viewed, processed and converted to TIFF format with the software FV10-ASW (Olympus Corporation, Tokyo, Japan). Assays were carried out with cells growing on a tissue culture dish (35-mm) with cover glass bottom (23-mm) plates. When E_2 or ICI 182780 was added, the solution was pipetted directly into the dishes, while pictures were taken simultaneously.

2.12. Statistical analysis

All data were analyzed and plotted utilizing GraphPad Prism version 4.00 for Windows software, (GraphPad Software, San Diego, CA, USA). Statistical

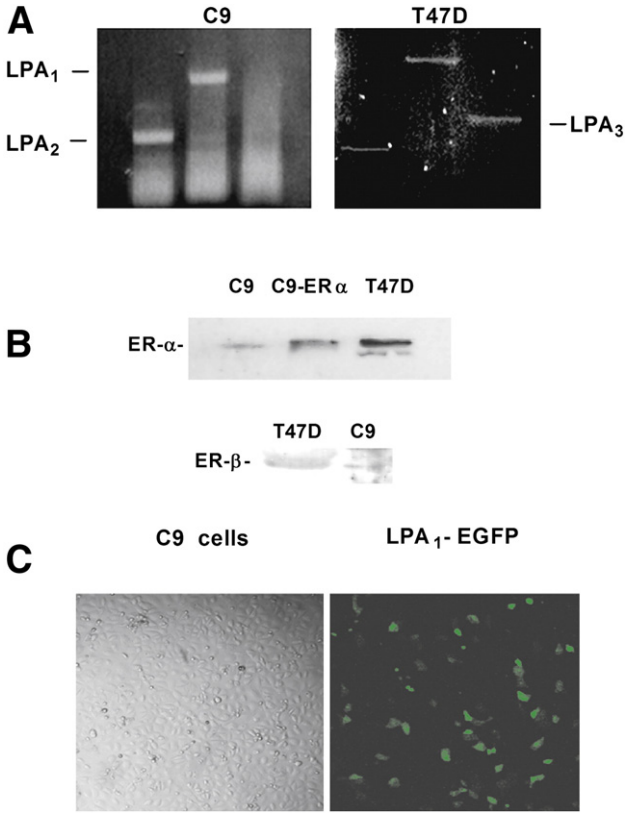

Fig. 1. LPA_1 and ERa expression in C9 cells. A) expression in C9 cells. A) LPA receptor subtype gene expression was characterized in C9 wild type cells and T47D cells by RT-PCR using specific primers. B) Western blot detection of ERα and ERβ in C9 cells stably expressing LPA_1–EGFP (C9), C9 cells transfected with ERα (C9-ERα) and wild type T47D cells. C) Confocal detection of C9 cells stably expressing LPA_1–EGFP receptors (LPA_1–EGFP). EGFP was excited utilizing the 488 nm line of a krypton/argon laser and fluorescence emitted detected with a 515–540 nm band pass filter.

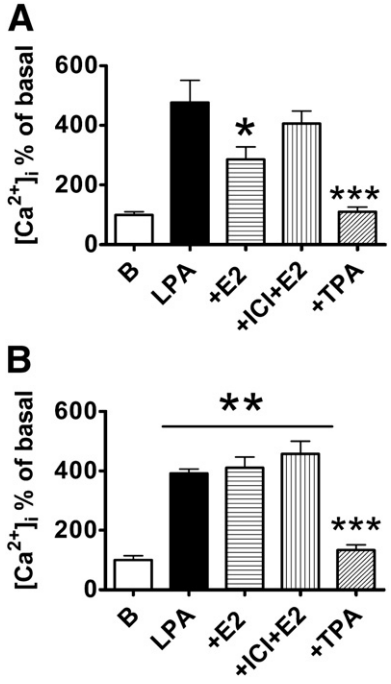

Fig. 2. Effect of E_2 and LPA on $[Ca^{2+}]_i$ in T47D and C9 wild type cells. T47D cells (A, upper panel) or C9 wild type cells (B, lower panel). Intracellular calcium in the absence of stimulus (B) or challenged with 1 μM LPA without pre-incubation (LPA) or pre-incubated with 1 μM E_2 (for 5 min) (+E_2); 2 μM ICI 182780 (20 min) and 1 μM E_2 (for 5 min) (+ICI+E_2) or with 1 mM TPA (+TPA) before challenging with LPA. Plotted are the means ± S.E.M. of 5 experiments. *$P \leq 0.05$ vs. all groups. **$p \leq 0.05$ vs. B. ***$p \leq 0.05$ vs. all groups except B.

analysis between comparable groups was performed using ANOVA with Bonferroni's posttest. A value of $p < 0.05$ was considered statistically significant, as stated in the figure legends.

3. Results

3.1. ERα and LPA_1 expression in C9 and T47D cells

The lack of subtype-selective LPA receptor antibodies that might reliably determine their expression in cells limited us to determine their gene expression by RT-PCR using specific primers for each subtype [45]. As shown in Fig. 1, C9 wild type cells express LPA_1, and LPA_2 receptor genes [44] and T47D cells express those for LPA_1, LPA_2 and LPA_3 receptors (Fig. 1A). C9 cells barely express ERα (Fig. 1B) and the expression of ERβ is either very low or absent since it was not clearly detected by Western blot analysis; T47D cells express both receptors (ERα: ERβ ratio approximately 9:1) [47] (Fig. 1B). We used C9 cells that stably express human LPA_1 fused to EGFP (LPA_1–EGFP) and transfected them with human ERα. Western blotting and confocal microscopy evidenced transient expression of ERα (Fig. 1B) and stable expression of LPA_1–EGFP (Fig. 1C) respectively.

3.2. Effect of 17-β estradiol on LPA_1 function and PKC and PI3K roles

The effect of LPA_1 was next examined. In T47D cells that abundantly expressed ERα, LPA induced an increase in $[Ca^{2+}]_i$, and cell-treatment with 17-β estradiol (E_2) for 5 min decreased the magnitude of the action of LPA (Fig. 2A); the effect of E_2 was abolished if cells were pre-incubated for 20 min with 2 μM ICI 182780, a selective estrogen antagonist (Fig. 2A). TPA was used as positive control of LPA_1 receptor desensitization (Fig. 2A and B). In C9 wild type cells, that barely express ERα, LPA also induced an increase in $[Ca^{2+}]_i$, but in contrast to that observed with T47D

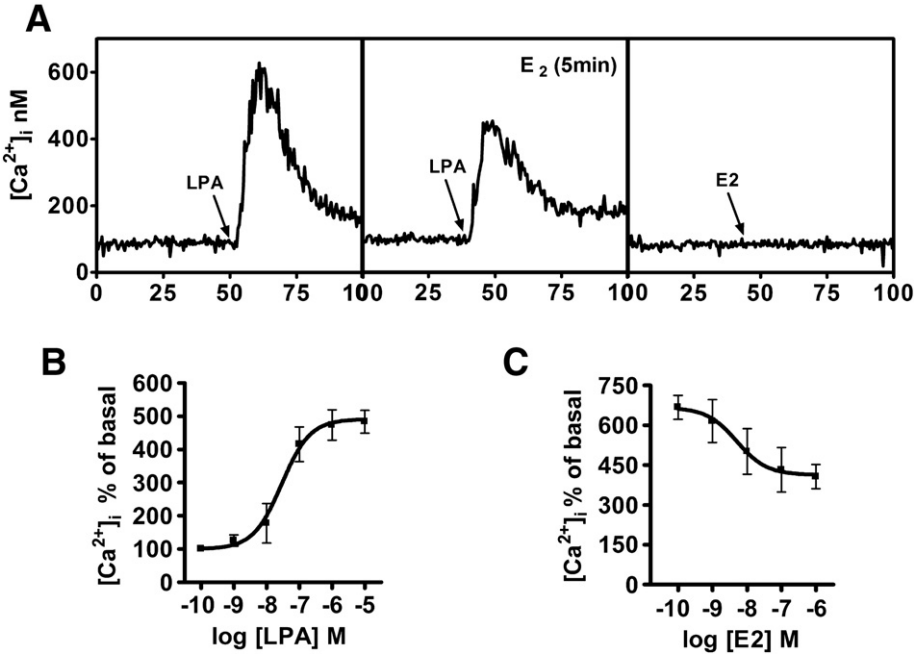

Fig. 3. Effect of different concentrations of lysophosphatidic acid (LPA) and 17-β estradiol (E_2) on intracellular free calcium concentration ($[Ca^{2+}]_i$). C9 cells stably expressing LPA_1–EGFP and transiently expressing ERα were labeled with fura-2AM. A) Representative tracings from cells challenged with 1 μM LPA (left), 1 μM E_2 (5 min)+LPA (center) or 1 μM E_2 (right) are shown. B) Cells were challenged with different concentrations of LPA. C) Cells were pre-incubated with the indicated concentrations of E_2 for 5 min and then challenged with 1 μM LPA. Plotted are means ± S.E.M. of 5 experiments performed with different cell preparations.

cells, this effect was not diminished by E_2 (Fig. 2B). As expected in C9 cells, that stably express LPA_1–EGFP and transiently express ERα, LPA increased intracellular calcium (Fig. 3A) with an EC_{50} value of 30 nM (Fig. 3B); cell incubation with E_2, for 5 min, decreased the effect of LPA, in a concentration-dependent fashion (Fig. 3C). Incubation with E_2 diminished (~30%) the increase in $[Ca^{2+}]_i$ induced by LPA (Fig. 3C and 4A) with an EC_{50} value of 5 nM. This effect of E_2 was abolished in cells pre-incubated (20 min) with the selective estrogen antagonist ICI 182780, (Fig. 4A). ICI 182780 neither induced any detectable change in basal $[Ca^{2+}]_i$ by itself nor altered the effect of LPA (Fig. 4A).

PKC and PI3K are key enzymes in heterologous desensitization and receptor phosphorylation [33,46–48]. To determine their role in E_2-induced LPA_1 desensitization, different PKC and PI3K inhibitors were tested. As shown in Fig. 4B, wortmannin (100 nM) and LY294002 (1 μM), blocked E_2 action on LPA-mediated increase in $[Ca^{2+}]_i$. Similarly, staurosporine a general inhibitor of PKC isoenzymes and Gö 6976, a selective PKC α inhibitor, abolished E_2-mediated modulation of LPA action (Fig. 4B). In a previous work [43], we found that rottlerin a selective inhibitor of PKC δ, abolished the effect of E_2 on α_{1b}-adrenergic mediated actions. Interestingly, rottlerin did not affect the desensitization of LPA_1 receptor, induced by E_2 (Fig. 4B), suggesting that PKC δ did not play a major role. None of these inhibitors alter the LPA-induced increase in $[Ca^{2+}]_i$ (supplemental Fig. 1).

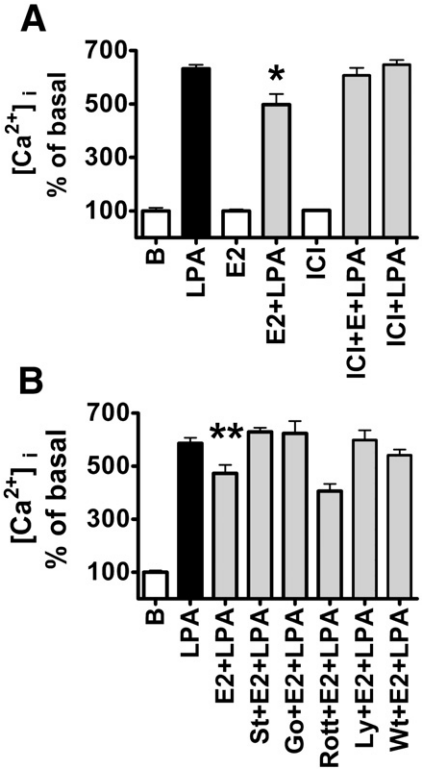

Fig. 4. Effect of E_2 and LPA on $[Ca^{2+}]_i$. Roles of PI3K and PKC. A) Cells were challenged with 1 μM LPA; 1 μM E_2; E_2 (5 min)+LPA; 2 μM ICI 182780; ICI 182780 (20 min)+E_2+LPA and ICI 182780 (20 min)+LPA. B) Cells were challenged with 1 μM LPA; 1 μM E_2 (5 min)+LPA; PKC general inhibitor: staurosporine (ST) 1 μM+E_2+LPA; PKC α inhibitor: Gö 6976 (Go) 1 μM+E_2+LPA; PKC δ inhibitor: rottlerin (Rott) 100 nM+E_2+LPA; PI3K inhibitors: LY294002 (LY) 1 μM or wortmannin (W) (100 nM)+E_2+LPA. Inhibitors were added 20 min before LPA or E_2. Plotted are means±S.E.M. of 5 experiments. *$P \leq 0.05$ vs. all groups. **$p \leq 0.05$ vs. all groups except Rott.

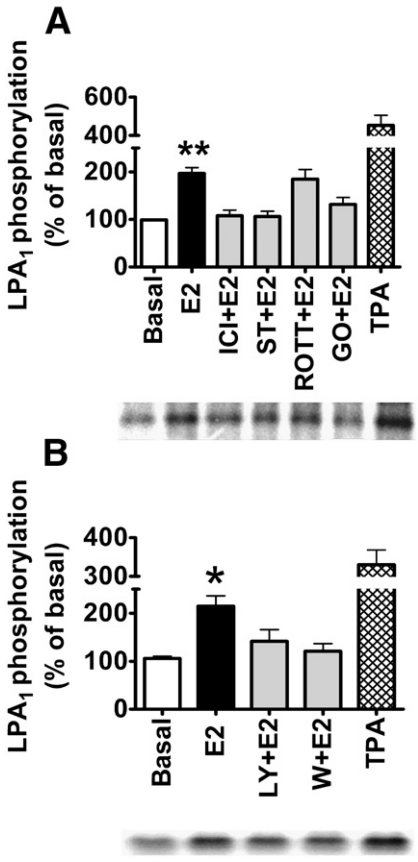

Fig. 5. Phosphorylation of LPA_1 induced by E_2, roles of PI3K and PKC. C9 stably expressing LPA_1–EGFP were metabolically labeled with [^{32}P]Pi. Cells were treated with E_2 for 5 min or pre-incubated 30 min prior to E_2 treatment with A) 2 μM ICI 182780 or PKC inhibitors or B) PI3K inhibitors. All inhibitor concentrations were employed as indicated in Fig. 3. Plotted are means±S.E.M. of 5 experiments. A representative autoradiograph is shown. *$P \leq 0.05$ vs. all groups. **$p \leq 0.05$ vs. all groups except Rott.

3.3. E_2 induces LPA_1 phosphorylation; PI3K and PKC participate in this event

We next examined the pathway through which E_2 induces LPA_1 receptor phosphorylation. Again TPA was used as a positive control (Fig. 5A). As shown in Fig. 5 (A and B), 1 μM E_2 increased this (~2-fold) and the estrogen receptor antagonist, ICI 182780, blocked this effect (Fig. 5A). Inhibitors such as staurosporine, Gö 6976, rottlerin, wortmannin and LY294002 were utilized to determine the roles of PKC and/or PI3K. As shown in Fig. 5A and B these inhibitors, with the exception of rottlerin, abolished E_2-induced LPA_1 receptor phosphorylation, suggesting that PKC α, but not δ, plays a role in this effect.

3.4. PKCα associates with LPA_1 in a dynamic fashion

To obtain further insight into the molecular events involved in these effects, associations of LPA_1 with PKC α, βI, βII, δ, ε and ζ were studied by co-immunoprecipitation. The results showed

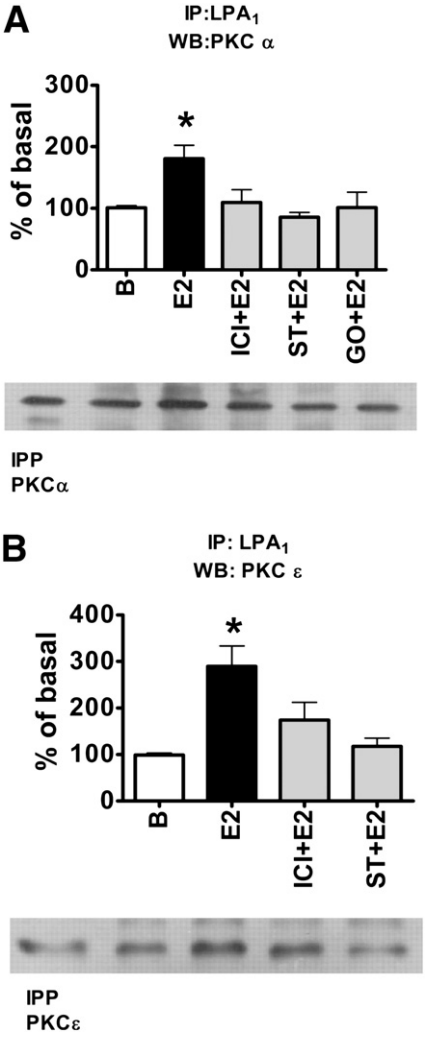

Fig. 6. Effect of E_2 on co-immunoprecipitation of LPA_1 with PKC a and PKC and PKC ε. C9 cells stably expressing LPA_1–EGFP were treated with 1 μM E_2 for 5 min or pre-incubated prior to E_2 treatment with 2 μM ICI 182780 or PKC inhibitors (concentrations as in Fig. 3) for 30 min. LPA_1 receptors were immunoprecipitated and samples were subjected to electrophoresis and Western blot analysis for PKC α (A, upper panel) or PKC ε (B, lower panel); IPP, samples immunoprecipitated with PKC isoform-selective antibodies. Representative Western blots are shown. Data are presented as % of basal. Plotted are means ± S.E.M. of 5 experiments performed using different cell preparations. *$P \leq 0.05$ vs. all groups.

that LPA_1 co-immunoprecipitated with PKC α, βI and ε isoforms under non-stimulated conditions (data not shown). Interestingly, isoforms whose co-immunoprecipitation increased in response to E_2 were PKC α (Fig. 6A) and PKC ε (Fig. 6B). Treatment with ICI 182780 diminished this effect (Fig. 6A). Staurosporine and Gö 6976 (Fig. 6A) blocked the association of PKC α with LPA_1. Western blotting of immunoprecipitated LPA_1 receptors was employed as loading control in co-immunoprecipitation experiments; the amount of immunoprecipitated receptor was essentially constant under all conditions tested (supplemental Fig. 2).

3.5. Subcellular localization of LPA_1 receptors

Current evidence indicates that GPCR phosphorylation is frequently associated with receptor internalization [27,29]. Therefore, our next aim comprised investigating whether E_2 could affect the subcellular localization of LPA_1. To study this, we used C9 LPA_1–EGFP cells transfected with ERα–ECFP. Transient expression of ERα–ECFP was corroborated by confocal microscopy (Fig. 7A and B). Fig. 7B shows that LPA_1–EGFP is located at the plasma membrane and in intracellular vesicles. E_2 induced internalization of LPA_1 receptors (Fig. 8A) and pre-treatment with ICI 182780 inhibited this effect (Fig. 8B). The process was visualized in real time showing a relatively rapid effect of E_2 (5 min) (supplemental Fig. 3 (movie)). To demonstrate that ERα–ECFP exerted the same effect on LPA_1 function, we tested the $[Ca^{2+}]_i$ response in cells C9 stably expressing LPA_1–EGFP. LPA clearly increased $[Ca^{2+}]_i$, E_2 decreased this effect and ICI 182780 inhibited the processes in a similar fashion to that observed in cells transfected with wild type ERα (Fig. 9).

4. Discussion

Phosphorylation and heterologous desensitization of LPA_1 receptors have been previously observed in response to agents acting though membrane receptors, and PKC plays a cardinal role in these effects [44]. Similarly, PKC mediates desensitization of α_{1b}-adrenoreceptors [48–50]. In the present paper, phosphorylation and heterologous desensitization of LPA_1 receptor by activation of an intracellular receptor are shown.

4.1. Estrogen modulates LPA_1 receptor function: roles of PI3K and PKC

Defining the manner in which estrogens might function is a complicated task, particularly because they bind to and activate two different intracellular nuclear receptors and may also have

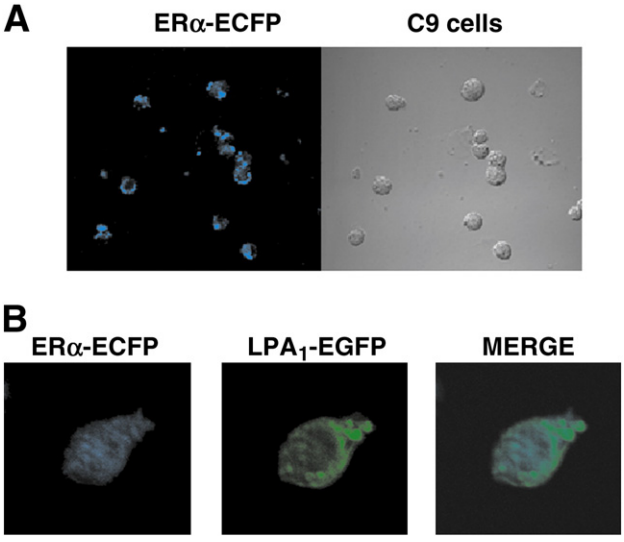

Fig. 7. ERαa–ECFP transfection and LPA_1–EGFP localization. C9 cells stably expressing LPA_1–EGFP were transfected with ERα–ECFP. A) ERα–ECFP expression in C9 cells. B) ER α–ECFP (left). LPA_1–EGFP (middle) was mainly localized at the plasma membrane and to a lesser extent in intracellular vesicles. EGFP was excited using the 488 nm line of a krypton/argon laser and the fluorescence emitted was detected with a 515–540 nm band pass filter. ECFP was excited using the 405 nm line of a krypton/argon laser and the fluorescence emitted was detected with a 425–476 nm band pass filter.

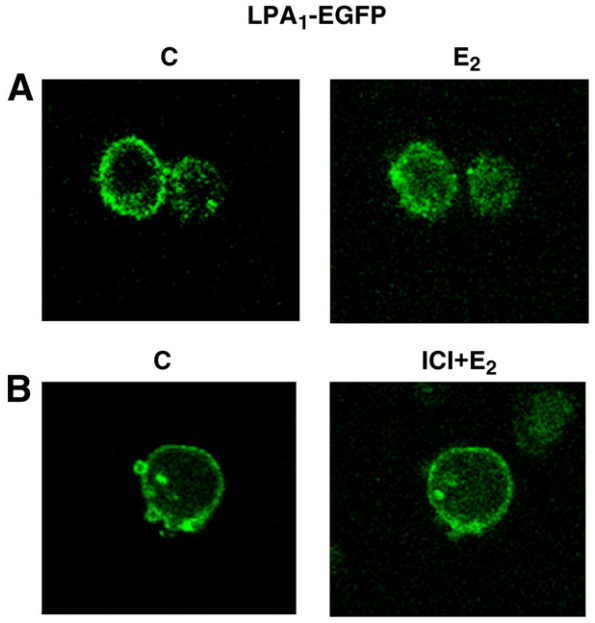

Fig. 8. Effect of E_2 on LPA_1 internalization. Cells were pre-incubated with no any agent (A, upper panels) or with 2 mM ICI 182780 for 30 min (B, lower panels) and further incubated for 5 min with no agent (C) or with 1 μM E_2. EGFP was excited using the 488 nm line of a krypton/argon laser and the emitted fluorescence was detected with a 515–540 nm band pass filter. Images are representative of 3 experiments using different cell preparations.

non-genomic actions through their interaction with GPCR (such as GPR30) [51,52]. Our present data clearly show participation of ERα in E_2-modulation of LPA_1 receptor function. In C9 wild type cells that poorly express ERα (Fig. 1B), E_2 does not modify the increase in $[Ca^{2+}]_i$ induced by LPA; it was not until ERα were overexpressed by transfection, that the effect of estrogen was evidenced (Figs. 2B, 3A and B). T47D cells that abundantly exhibit ERα (Fig. 1B) show desensitization of LPA receptors in response to E_2 treatment (Fig. 2A). The hardly detectable presence of ERβ in C9 and its low expression in T47D cells (Fig. 1B) further suggest that E_2 effects were mediated through ERα. Nevertheless, we cannot completely rule out the participation of these intracellular receptors or that of GPCRs in these actions.

Modulation of membrane receptor function by ERα has been documented in different studies [43,53,54]. Our previous work showed that α_{1b}-adrenoreceptors are regulated by ERα [43] by inducing desensitization, phosphorylation, and internalization. Kelly and coworkers [53,54] have shown that ERs can modulate the action of other GPCRs, such as μ-opiod and GABA-B receptors, by uncoupling these to the rectifying K^+ channels through protein kinase A and PKC activities. A recent study demonstrated effects of E_2 through transactivation of EGF receptor via stimulation of sphingosine kinase 1 activity, release of sphingosine 1-phosphate and autocrine action on SP1 receptors [55]. Our data shows that E_2, through ERα, induces LPA_1 phosphorylation, desensitization and internalization in non-genomic rapid fashion.

Different studies indicate that the fast effects of estrogens are mediated by a subpopulation of conventional plasma membrane-associated ER [56,57]. ERα fused to the green fluorescent protein has been used to visualize ERα in the plasma membrane of neurites [57]; it is possible that a small population of this receptor, in proximity to the plasma membrane, might participate in the rapid effects of E_2 presented herein.

Association of ERα with the PI3K p85α regulatory subunit resulting in PI3K/Akt signaling pathway activation is among the non-genomic mechanisms of action of estrogens [40–42]. We used two blockers of PI3K with different mechanisms of action; wortmannin, a potent and selective non-competitive inhibitor [58] and LY 294002, a blocker that competes at the ATP binding site of this kinase [59]. These inhibitors, clearly and consistently, blocked E_2-induced LPA_1 receptor desensitization and phosphorylation (Figs. 4B and 5B), which strongly suggested a role of PI3K in these effects.

PI3K regulates PKC activity through the synthesis of 3-phosphorylated phosphoinositides, which directly interact with PKC. In addition, they are important regulators of phosphoinositide-dependent protein kinase 1 (PDK-1), which phosphorylates the activation loop of some PKC isoforms [60]. In our previous work, it was shown that ERα associates with and stimulates PI3K activity, leading to PKC δ activation, which phosphorylates and desensitizes α_{1b}-ARs [43]. In this work inhibitors of PKC and PI3K blocked E_2 effects on LPA_1 receptor desensitization and phosphorylation, suggesting that they act sequentially and not in parallel. It is very likely that PI3K activation may precede that of PKC, because phorbol esters induce marked LPA_1 receptor phosphorylation even in the presence of PI3K inhibitors. Nevertheless, it should be mentioned that PI3K actions, probably extend beyond inducing PKC activation. PI3K is a dual kinase capable of phosphorylating phosphoinositides and proteins and it was shown recently that it plays key roles in β_2-AR endocytosis [61]. Interestingly, PI3K serine/threonine protein kinase activity phosphorylates tropomyosin, this action appearing to be critical for receptor internalization [61]. However, it remains to be shown whether a similar role is played for LPA_1 receptor internalization.

PKC activation blocks LPA_1 actions by inducing its phosphorylation [44]. PKC α association with LPA_1 was consistent with the inhibitory ability of staurosporine and Gö 6976 over LPA_1 phosphorylation and desensitization (Fig. 6A). It has been known for some time that formation of protein complexes

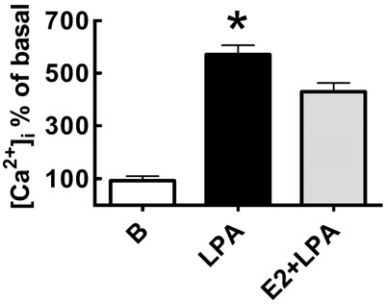

Fig. 9. $[Ca^{2+}]_i$ determination in C9 cells stably expressing LPA_1–EGFP and transfected with ERa–ECFP. Cells were pre-incubated in the absence of any agent or with 1–ECFP. Cells were pre-incubated in the absence of any agent or with 1 μM E_2 for 5 min. After this pre-incubation cells were challenged with 1 μM LPA. Plotted are means±S.E.M. of 3 experiments. Data are present as % of basal $[Ca^{2+}]_i$. *$P \leq 0.05$ vs. all groups.

comprises a central event in the signaling and regulation of receptors with intrinsic tyrosine kinase activity [62]. At present, it is accepted that this is a more general process, and not solely limited to these receptors [63]. Formation of multimolecular complexes has been shown to participate in the regulation of GPCR, including among others, α_{1b} adrenoceptors [43] and angiotensin AT_1 receptors [64]. The term signalplex has been coined to define these protein–protein multimolecular complexes involved in signaling and receptor modulation [65]. Protein–protein interactions are defining new connections among signaling pathways, these are potentially capable of controlling spatial and temporal cell responses [63–66]. The data presented herein suggest that the association of PKC α and LPA_1 receptors may be part of a signalplex involved in LPA_1 modulation by estrogens. It is currently unknown whether such a possible interaction may occur directly or via anchoring or scaffolding proteins.

In the present work, the PKC isoforms α, βI, δ and ε co-immunoprecipitated with LPA_1 under non-stimulated conditions; nonetheless, treatment with E_2 only increased the association of PKC α and ε with the LPA_1 receptor. Our data showed that association of PKC α with LPA_1 receptors after E_2 treatment, correlated with desensitization and phosphorylation of these receptors; it appears likely that PKC α increases its association with LPA_1 receptors, in response to E_2, to induce phosphorylation and consequently desensitization. Notwithstanding this, the role of PKC ε association with LPA_1 receptors remains uncertain.

The existence of five LPA receptor subtypes suggests that these might have different properties in their action and/or regulation; additionally, it is possible that combinatorial functions or synergisms may exist in cells expressing more than one subtype. LPA receptor phosphorylation and desensitization might have physiological importance; given that receptor's persistent activity could participate in the genesis of pathological conditions. Information concerning LPA_1 receptor tissue expression and regulation appears to be important in establishing differences among receptor properties and functions.

The tissue distribution and regulation of LPA receptor subtypes is beginning to be unraveled. To the best of our knowledge no previous information regarding estrogen-mediated regulation of LPA receptor function has been reported. Considering the plethora of effects in which these are involved [1,3–5], it is likely that modulation of their expression and function by estrogens could play roles in physiological or pathological conditions, particularly in estrogen-responsive tissues. For instance, it has been shown that LPA plays a pivotal role in embryo implantation via LPA_3 receptors [67], and additional evidence indicates that estrogens and progesterone modulate their expression in the uterus [68]. On the pathological side, it is known that serum LPA is elevated in patients suffering from ovarian cancer and it has been suggested that it might serve as a biomarker for the disease [69,70]. This type of cancer cells expresses LPA_1 and LPA_2 receptors [71]. Therapeutic intervention at the levels of LPA generation, LPA receptor expression, signaling, and regulation, appears promising for patients suffering from this disease. We think that our data showing that E_2 through ERα induces LPA_1 receptor phosphorylation, desensitization and internalization, with the participation of PI3K and PKC, contribute to understanding the tight interaction between signaling pathways in health and disease.

In summary, the present data show that E_2 through ERα induces LPA_1 receptor phosphorylation, desensitization, and internalization, and that PI3K and PKC α play a cardinal role in these actions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2007.11.014.

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